APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1997, p. 3561–3568 Vol. 63, No. 9
0099-2240/97/$04.00+0

Microbial Utilization of Free and Clay-Bound Insecticidal Toxins from *Bacillus thuringiensis* and Their Retention of Insecticidal Activity after Incubation with Microbes

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Received 3 June 1997/Accepted 3 July 1997

The insecticidal toxins produced by *Bacillus thuringiensis* subspp. *kurstaki* and *tenebrionis* were resistant when bound on clays, but not when free, to utilization by pure and mixed cultures of microbes as sources of carbon and carbon plus nitrogen, and their availability as a nitrogen source was reduced. The bound toxins retained insecticidal activity both before and after exposure to microbes or pronase. The insecticidal activity of the toxins persisted for 40 days (the longest time evaluated) in nonsterile soil continuously maintained at the −33-kPa water tension and room temperature, alternately air dried and rewetted to the −33-kPa water tension, or alternately frozen and thawed, although alternate drying and wetting reduced the activity.

Subspecies of *Bacillus thuringiensis* produce insecticidal, proteinaceous, parasporal protoxins that are active against the larvae of the orders Lepidoptera, Coleoptera, and Diptera, as well as against other groups of animals (18). The proteins are produced as inactive protoxins that are activated in the larval midgut to the insecticidal toxins by solubilization in the high pH (ca. 10.5) and cleavage by specific proteases (2, 31). The protoxins are insoluble at low pH, with the exception of the CryIII protoxin, which is also soluble at low pH (35). The active toxins interact with receptors, which presumably confers specificity, on the epithelial cells of the larval midgut, where the toxins form pores and destroy the cells by colloidal osmotic lysis (2, 69, 76). Receptors are also present in the larvae of nontarget insects but apparently in lower numbers (31, 68, 69), although they can be present in high numbers in some nonsusceptible larvae (24, 76).

Truncated forms of the genes that code for these toxins have been genetically engineered into plants and other bacteria that express the active toxins rather than the inactive protoxins. Because the active toxins do not require solubilization and proteolytic cleavage, two of the barriers that are involved in specificity are removed. Thus, beneficial insects, as well as organisms at higher trophic levels, could be harmed (3, 22, 32, 33). After commercially usable portions of transgenic plants have been harvested, the rest of the plant biomass will be plowed into soil, where the toxins will bind on clays (61) and humic substances (13) and become resistant to microbial degradation.

The toxins from *B. thuringiensis* subsp. *kurstaki* (CryI protein) and subsp. *tenebrionis* (CryIII protein) bound rapidly on montmorillonite homoionic to K, Na, Ca, Mg, La, or Al; on kaolinite homoionic to Na or Ca; on "dirty" montmorillonite and kaolinite coated with two types of polymeric oxyhydroxides of Fe(III); and on the clay-size, but not on the silt- and sand-size, fraction separated from various soils. Some intercalation of montmorillonite occurred with both toxins, although the toxins did not completely penetrate the clay (61). The toxins, free or bound, were detected by a dot blot enzyme-linked immunosorbent assay and remained insecticidal to the larvae of the tobacco hornworm (*Manduca sexta*) (*B. thuringiensis* subsp. *kurstaki* toxin) and the Colorado potato beetle (*Leptinotarsa decemlineata*) (*B. thuringiensis* subsp. *tenebrionis* toxin) for more than 40 days after addition to nonsterile soil (58, 59). Similar results were obtained with the toxin from *B. thuringiensis* subsp. *kurstaki* and humic acids extracted from different soils (13).

These insecticidal proteins may be removed from or inactivated in the environment by (i) consumption by insect larvae, (ii) degradation and eventual mineralization by microorganisms, or (iii) sunlight. When genes that code for the active toxins are expressed by transgenic plants (1, 5, 21, 65–67) and microorganisms (8, 16, 36, 41–45, 50–52, 62, 63) that are indigenous or adapted to natural environments wherein they continue to grow and synthesize the toxins, the toxins may accumulate. Hence, the levels of active toxins in soil could be greater and be present longer than those introduced by periodic spraying of commercial preparations of *B. thuringiensis* containing protoxins and could exceed consumption, inactivation, and degradation. This could result in sufficiently high concentrations of the toxins to select toxin-resistant target organisms (4, 6, 19, 20, 29, 55–57, 68, 69) and constitute a hazard to nontarget organisms (3, 10, 33), especially if some of the toxins are bound on soil constituents.

There have been some studies of the persistence of the cells and spores of *B. thuringiensis* in soil (17, 27, 46, 48, 72–75), but there have been few investigations to determine the persistence and rate of inactivation of the insecticidal proteins in soil (46, 47, 58–61). The persistence of the toxin from *B. thuringiensis* subsp. *kurstaki*, determined by bioassay, exceeded 7.5 months (the longest time evaluated) and appeared to be related to the pH of the soils (60). Palm et al. (47), using a chemical extraction procedure and an enzyme-linked immunosorbent assay, detected the toxin from *B. thuringiensis* subsp. *kurstaki* for up to 140 days (the longest time evaluated) in soil amended with transgenic cotton or pure toxin. The present study indicates that the toxins from *B. thuringiensis* subsp. *kurstaki* and subsp. *tenebrionis* become resistant to microbial

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utilization and proteases and remain insecticidal when bound on clay minerals and the clay-size fraction from soil.

MATERIALS AND METHODS

Microorganisms and cultural conditions. *Proteus vulgaris*, *Enterobacter aerogenes*, and a mixed culture of bacteria containing predominantly *Agrobacterium* spp. from a contaminated suspension of protoxin from *B. thuringiensis* subsp. *kurstaki* were maintained on slants of tryptic soy agar (Difco) and subcultured for 48 h before use. Mixed microbial cultures were obtained from several soils as follows. (i) A protein-enriched soil slurry {10 g of soil added to 50 ml of modified Davis citrate minimal medium (DCMM [25]; 7 g of K_2HPO_4, 2 g of KH_2PO_4, 0.04 g of Na-citrate · $2H_2O$, 0.1 g of $MgSO_4 \cdot 7H_2O$, and 0.5 g of NH_4NO_3 in 1 liter of distilled water [dH_2O]; pH 7.2) containing a mixture of gelatin, casein, pepsin, and lactoglobulin at a total protein concentration of 1 mg/ml} was shaken (120 rpm) at 28°C and diluted (1:10) every 24 h with protein-amended DCMM for 5 days. The final culture contained both bacteria and fungi, as determined microscopically and by plating on tryptic soy agar and Sabouraud-dextrose agar. (ii) A freshly collected garden soil (10 g) was suspended in 50 ml of DCMM (without NH_4NO_3), the soil was removed by centrifugation at 480 × *g*, the cells in the supernatant were concentrated at 20,200 × *g*, and the pellet was resuspended in DCMM to an optical density at 420 nm (OD_{420}) of 0.9 to 1.0 and used immediately as an inoculum. (iii) Another freshly collected garden soil was suspended in tap water (1 g of soil–4 ml of H_2O) and used immediately as an inoculum. *Streptomyces griseus* (a producer of pronase E) and *Streptomyces hygroscopicus* (a producer of neutral proteases) were maintained on yeast extract-malt extract agar (YEM), which was composed of 4 g of yeast extract (Difco), 10 g of malt extract (Sigma), 4 g of glucose, and 20 g of agar in 1 liter of dH_2O (pH 7.2). (The organisms and the composition of the medium were kindly provided by David P. Labeda, U.S. Department of Agriculture, Peoria, Ill.)

The pure, mixed bacterial, and mixed microbial (from the protein-enriched soil slurry) cultures were conditioned to grow on pepsin or the toxin from *B. thuringiensis* subsp. *kurstaki* (200 μg/ml) for 5 days, with the cultures being diluted daily with fresh DCMM containing 200 μg of either protein per ml. The cultures were washed four times (10,000 × *g*) with phosphate-buffered saline (pH 7.0) and resuspended in DCMM to an OD_{420} of 0.9 to 1.0.

Preparation of clays. The <2-μm-particle-size fraction of montmorillonite, a 2:1, Si-Al, swelling clay, and kaolinite, a 1:1, Si-Al, nonswelling clay, were purified from bentonite and kaolin (Fisher Scientific Co.), respectively, and made homoionic to various cations (14, 28).

Separation of the clay-size fraction from soil. The clay-size fraction (<2-μm effective diameter) from Kitchawan soil (K-soil) (a sandy loam soil collected at the Kitchawan Research Laboratory of the Brooklyn Botanic Garden, Ossining, N.Y. [60]), unamended or amended to 6% (vol/vol) with kaolinite (K6K-soil) or montmorillonite (K6M-soil), was separated by sedimentation according to Stokes' Law (15, 58, 59).

Purification of toxins. The toxin from *B. thuringiensis* subsp. *kurstaki* (66 kDa) was purified from Dipel 2× (Abbott Laboratories) by extraction with MOPS buffer (0.1 M 3-*N*-morpholinopropane-sulfonic acid buffer [pH 7.8] containing 1 M KSCN and 0.5 M dithiothreitol) (61, 70). The toxin from *B. thuringiensis* subsp. *tenebrionis* (68 kDa) was purified from commercial M-One (Mycogen Corporation) with 1 M Na_2CO_3 (39, 58, 59). The protein solutions were sequentially filtered through sterile 0.45- and 0.22-μm-pore-diameter filters (Millipore) and stored at −20°C.

Binding of the toxins on clay minerals. The protein concentration of solutions of the toxins was determined by the Lowry method (37), with bovine serum albumin as the standard. The toxins, suspended in 0.1 M phosphate buffer (pH 8.0), were mixed with the homoionic clays or the clay-size fraction from the unamended or clay-amended soil, suspended in dH_2O adjusted to pH 6.0, and rotated on a motorized wheel (70 rpm) at 24 ± 2°C. The mixtures were then centrifuged at 34,800 × *g*, and the amount of protein in the supernatant was subtracted from the amount added initially, to determine the amount of protein adsorbed at equilibrium (61). The clay-protein complexes were then washed with dH_2O, with centrifugation, until no protein was detected in the supernatants (two to three washes), followed by three more washes. The total amount of protein recovered in the equilibrium supernatant and in all washes was subtracted from the amount added initially, to determine the amount of protein bound (61, 70, 71).

Utilization of free and clay-bound toxins. The utilization of the toxins, either free or bound, as a sole source of carbon by the pure and mixed cultures was determined by growth in DCMM. Tubes containing DCMM (3 ml) amended with pepsin (which was readily used by all cultures and, therefore, was used as a control), free toxins, or clay-bound toxins (all at 100 to 500 μg/ml of protein) were inoculated with either pure or mixed cultures (0.3 ml) and rotated (90 rpm) at 28 or 37°C. Changes in OD_{420} were measured hourly for the first 8 h and then at longer intervals. The experiments were done in triplicate and repeated at least twice. The data are expressed as means ± the standard error of the means.

Growth of the cultures on the toxins, free or bound, as a sole source of carbon, nitrogen, or carbon plus nitrogen was also determined by the direct Warburg method of O_2 uptake with a Gilson respirometer (64). The Warburg flasks contained the following: (i) 2.5 ml of DCMM, appropriately amended so that pepsin (control) or the toxin from *B. thuringiensis* subsp. *kurstaki* or *tenebrionis*, free or bound, was the sole source of carbon, nitrogen, or carbon plus nitrogen (DCMM without NH_4NO_3 was used with the latter two variables) in the main compartment; (ii) 0.5 ml of a culture (OD_{420} of 0.9 to 1.0) previously conditioned on pepsin or the toxin from *B. thuringiensis* subsp. *kurstaki* and washed four times with sterile phosphate-buffered saline in the sidearm of the flask; and (iii) a folded filter-paper wick saturated with 200 μl of 20% KOH in the center well to absorb CO_2. After equilibration for 30 min, the culture was introduced to the main compartment, and measurements of O_2 uptake were made hourly or half-hourly at 25 or 37°C until a plateau or a decrease in O_2 consumption occurred. The experiments were done in duplicate and repeated at least twice. The data are expressed as means ± the standard error of the means.

Assay of the insecticidal activity of the toxin from *B. thuringiensis* subsp. *kurstaki* against *Manduca sexta*. Dilutions (100 μl) in dH_2O of free toxin or equivalent amounts of toxin bound on montmorillonite or kaolinite homoionic to Na or on the clay-size fraction from the K-, K6K-, or K6M-soil, before and after exposure to pure or mixed cultures or to pronase E (1 mg/ml [Sigma]), were pipetted onto 5 ml of solidified tobacco hornworm medium (Carolina Biological Co.) in vials (3 cm in diameter by 6 cm in height) and allowed to dry (58–60). Four second-instar larvae of *M. sexta*, hatched from eggs (Carolina Biological Co.), were placed into replicate vials and incubated at 27 ± 2°C under light, and mortality was evaluated after 48, 72, and 120 h. The lethal concentration to kill 50% of the larvae (LC_{50}) was determined from the 120-h data by the PC-POLO probit procedure (49) (Le Ora Software, 1987). The experiments were done in duplicate and repeated at least twice. The relative potency (RP) of the bound toxin was determined for each experiment: the LC_{50} of the free toxin was divided by the LC_{50} of the bound toxin of the same treatment. An RP of 1.0 indicates no difference in potency between free and bound toxin, an RP of >1.0 indicates a greater potency of bound toxin, and an RP of <1.0 indicates a lower potency of bound toxin.

The toxin from *B. thuringiensis* subsp. *kurstaki* (100 μg), free or bound on montmorillonite or kaolinite homoionic to Na, was also added to nonsterile K-soil (1 g), and the soil was then incubated for 40 days. There were three treatments of this soil. (i) The soil was continuously maintained at room temperature at the −33-kPa water tension in a high-humidity chamber. (ii) The soil was maintained at room temperature at the −33-kPa water tension for 7 days, air dried for 7 days, and rewetted and maintained at the −33-kPa water tension for 7 days, and then the cycle was repeated. (iii) The soil was maintained at room temperature at the −33-kPa water tension for 7 days and then at −20°C for 7 days, and the cycle was repeated three times. Dilutions (100 μl) of the soil made with dH_2O were assayed as described above. The experiment was done in duplicate.

RESULTS

Utilization of free and clay-bound toxin as a sole source of carbon, nitrogen, or carbon plus nitrogen. The free toxin from *B. thuringiensis* subsp. *kurstaki* or *tenebrionis* was readily utilized as a carbon source by a mixed microbial culture from the protein-enriched soil slurry, and there were no significant differences in utilization between cultures conditioned on pepsin and those conditioned on the toxin from *B. thuringiensis* subsp. *kurstaki* (Fig. 1). Consequently, subsequent inocula were conditioned only on pepsin because the purification of the toxin was time-consuming and expensive. The toxin from *B. thuringiensis* subsp. *kurstaki* or *tenebrionis* was not inhibitory to the growth of the mixed culture, and growth was better on the toxin from *B. thuringiensis* subsp. *kurstaki* than on that from *B. thuringiensis* subsp. *tenebrionis* or on pepsin.

P. vulgaris and *E. aerogenes* utilized the toxin from *B. thuringiensis* subsp. *kurstaki* when free but not when bound on montmorillonite homoionic to Ca (Fig. 2A and B). Cultures from the protein-enriched soil slurry, freshly collected garden soil, or the contaminated suspension of protoxin from *B. thuringiensis* subsp. *kurstaki* also readily utilized the toxin when free but not when bound on montmorillonite homoionic to Ca (Fig. 2C and D) or the clay-size fraction from the K-soil (Fig. 3A and B and Table 1). When 1 mg of montmorillonite homoionic to Ca per ml (Fig. 2C) or the clay-size fraction (Fig. 3B) was added to cultures growing on pepsin, growth was reduced, probably because some pepsin bound on the clays and was rendered unavailable.

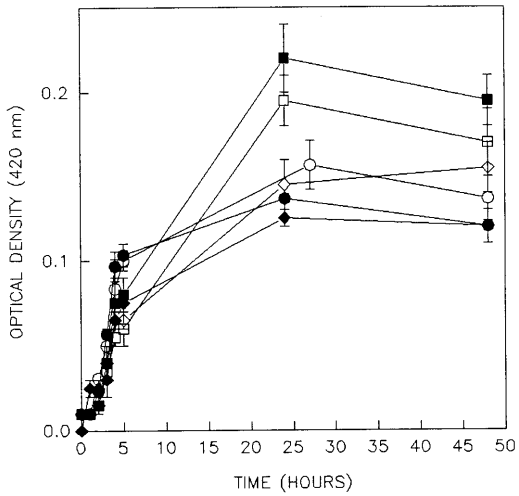

FIG. 1. Growth of a mixed microbial culture from a protein-enriched soil slurry on pepsin (200 μg/ml [● and ○]), toxin from *B. thuringiensis* subsp. *kurstaki* (200 μg/ml [■ and □]), or toxin from *B. thuringiensis* subsp. *tenebrionis* (200 μg/ml [◆ and ◇]) after being conditioned on either the toxin from *B. thuringiensis* subsp. *kurstaki* (closed symbols) or pepsin (open symbols) for the induction of proteases. Data are expressed as means ± the standard error of the means, which is indicated when not within the dimensions of the symbols.

The inability of the microbes to utilize the bound toxins as a carbon source was confirmed by measurements of O_2 uptake (Table 2). The toxin from *B. thuringiensis* subsp. *kurstaki* was readily utilized by cultures from (i) the protein-enriched soil slurry when free but not when bound on montmorillonite or

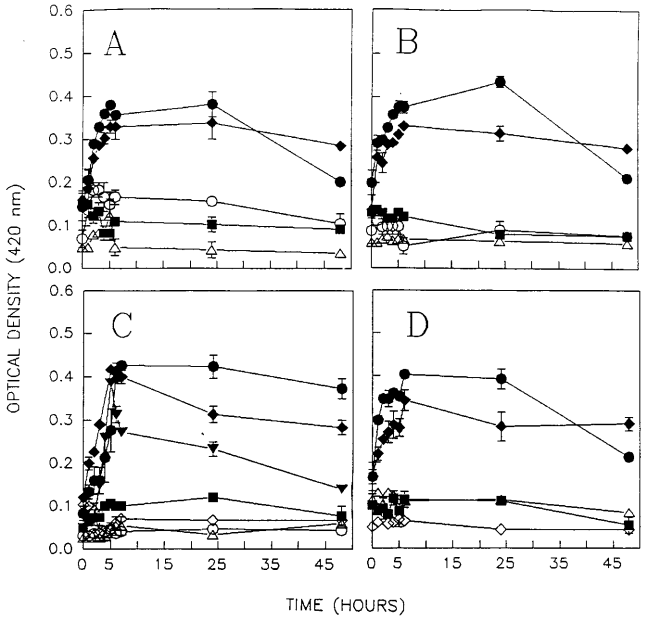

FIG. 2. Growth of (A) *P. vulgaris*, (B) *E. aerogenes*, (C) a mixed microbial culture from a protein-enriched soil slurry, or (D) a mixed bacterial culture from a suspension of the protoxin from *B. thuringiensis* subsp. *kurstaki* on pepsin (500 μg/ml) or the toxin from *B. thuringiensis* subsp. *kurstaki* (500 μg/ml), free or bound on montmorillonite homoionic to calcium. Data are expressed as means ± the standard error of the means, which is indicated when not within the dimensions of the symbols. ◆, pepsin; ▲, pepsin plus montmorillonite (1.00 mg/ml); ●, toxin; ■, toxin bound on montmorillonite (1.40 mg/ml); △, control (no toxin, no clay, microbes); ○, control (pepsin, no microbes); ◇, control (1.00 mg of montmorillonite per ml, no microbes).

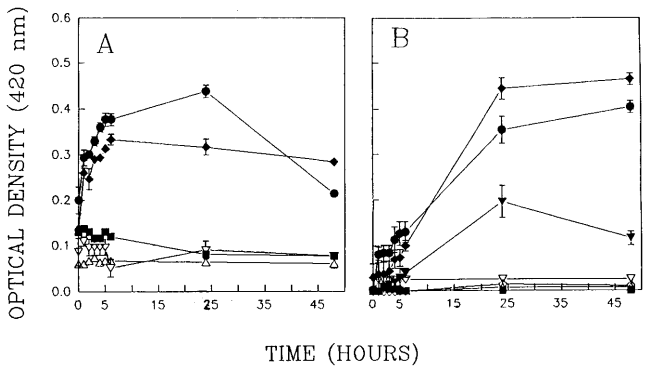

FIG. 3. Growth of a mixed microbial culture from a protein-enriched soil slurry (A) or a nonenriched soil slurry (B) on pepsin (200 μg/ml) or the toxin from *B. thuringiensis* subsp. *kurstaki* (200 μg/ml), free or bound on the clay-size fraction separated from K-soil. Data are expressed as means ± the standard error of the means, which is indicated when not within the dimensions of the symbols. ◆, pepsin; ▼, pepsin plus clay-size fraction (1.00 mg/ml); ●, toxin; ■, toxin bound on clay-size fraction (0.66 mg/ml); △, control (no toxin, no clay, no microbes); ◇, control (no toxin, no clay, microbes); ▽, control (no toxin, 1.00-mg/ml clay-size fraction, microbes).

kaolinite homoionic to Ca or Na (Fig. 4A and B) or on the clay-size fraction from the K-, K6K-, or K6M-soil (Fig. 4C) and (ii) the 1:4 dilution of a fresh garden soil when free but not when bound on montmorillonite and kaolinite homoionic to Na (Fig. 5A) or the clay-size fractions (Fig. 5B). The toxin from *B. thuringiensis* subsp. *tenebrionis* was also utilized by the culture from the protein-enriched soil slurry when free but not when bound on montmorillonite homoionic to Na or the clay-size fraction from the K-soil (Fig. 6).

The free toxin from *B. thuringiensis* subsp. *kurstaki*, in both the presence and absence of glucose (255 μg/ml), was readily utilized by the culture from the protein-enriched soil slurry as a source of carbon, nitrogen, and carbon plus nitrogen. However, the toxin was not utilized as a source of carbon or carbon plus nitrogen when bound on montmorillonite and kaolinite homoionic to Na (Fig. 7A) or the clay-size fractions (Fig. 7B). The bound toxin was utilized as the source of nitrogen but to a lesser extent than when free (Fig. 7), and it was utilized more when bound on the homoionic clays than on the clay-size fractions (Fig. 7).

Insecticidal activity of clay-bound toxin from *B. thuringiensis* subsp. *kurstaki* before and after exposure to microbes. The toxin bound on montmorillonite and kaolinite homoionic to Na or the clay-size fractions remained toxic to the second-instar larvae of *M. sexta* both before and after exposure to microbes or pronase E for 3 or 7 days (Table 3). Neither the clays alone nor the microbes were toxic to the larvae. The bound toxin incubated with microbes or pronase had RPs that were greater than 1.0, indicating again that binding of the toxin on clay protected it from being inactivated. The toxin bound on the clay-size fractions and incubated without microbes for 7 days was less insecticidal than the free toxin or the toxin bound on the homoionic clays, probably because the amounts of toxin bound on the clay-size fractions were smaller than those on the homoionic clays, and the larvae had to ingest more of the fractions to obtain an amount of toxin equivalent to that bound on the homoionic clays.

The RPs of the toxin bound on montmorillonite and kaolinite homoionic to Na and incubated continuously in nonsterile soil for 40 days at the −33-kPa water tension and room temperature were 1.8 and 1.4, respectively, indicating that the bound toxin was also protected from inactivation in soil (Table

TABLE 1. Utilization of the toxins from *B. thuringiensis* subspp. *kurstaki* and *tenebrionis*, free or bound on homoionic montmorillonite or the clay-size fraction from soil, by various microbial cultures

Organism(s)[a]	Toxin	Clay or soil fraction bound[b]	Growth on toxin[c]	
			Free	Bound
P. vulgaris	*B. thuringiensis* subsp. *kurstaki*	M-Ca	+	−
E. aerogenes	*B. thuringiensis* subsp. *kurstaki*	M-Ca	+	−
Mixed contaminant culture from a suspension of *B. thuringiensis* subsp. *kurstaki* protoxin (mainly *Agrobacterium* sp.)	*B. thuringiensis* subsp. *kurstaki*	M-Ca	+	−
Mixed culture from a protein-enriched soil slurry	*B. thuringiensis* subsp. *kurstaki*	M-Ca	+	−
		M-Na	+	−
		M-Al	+	−
		K-soil	+	−
		K6K-soil	+	−
		K6M-soil	+	−
	B. thuringiensis subsp. *tenebrionis*	M-Ca	+	−
		M-Na	+	−
		M-Al	+	−
		K-soil	+	−
		K6K-soil	+	−
		K6M-soil	+	−
Mixed culture from a garden soil	*B. thuringiensis* subsp. *kurstaki*	M-Ca	+	−
		M-Na	+	−
		M-Al	+	−
		K-soil	+	−
		K6K-soil	+	−
		K6M-soil	+	−

[a] All cultures were incubated at 37°C, except for the mixed culture from garden soil, which was incubated at 28°C.
[b] M-Na, montmorillonite homoionic to Na; M-Ca, montmorillonite homoionic to Ca; M-Al, montmorillonite homoionic to Al; K-soil, clay-size fraction from Kitchawan soil; K6K-soil, clay-size fraction from Kitchawan soil amended to 6% (vol/vol) with kaolinite; K6M-soil, clay-size fraction from Kitchawan soil amended to 6% (vol/vol) with montmorillonite.
[c] +, growth; −, no growth. Utilization was determined by growth measured at 420 nm.

4). The RPs of the bound toxin incubated in soil that was alternatively frozen and thawed were 1.8 and 3.2, respectively, indicating that the toxin was not released from the clays and rendered available for biodegradation by this treatment. In contrast, alternate drying and wetting reduced both RPs to 0.7, indicating that this treatment rendered the bound toxin more susceptible to biodegradation. Although the latter two treatments reduced the activity of both free and bound toxin (i.e.,

TABLE 2. Utilization of the toxin from *B. thuringiensis* subsp. *kurstaki*, free or bound on homoionic clay minerals or the clay-size fraction from soil, by various microbial cultures

Source of organisms	Clay or soil fraction bound[a]	Utilization of toxin as source of[b]:					
		Carbon		Nitrogen		Carbon and nitrogen	
		Free	Bound	Free	Bound	Free	Bound
Mixed culture from a protein-enriched soil slurry[c]	M-Na	+	−	+	+	+	−
	M-Ca	+	−				
	M-Al	+	−				
	K-Na	+	−	+	+	+	−
	K-Ca	+	−				
	K-soil	+	−	+	+	+	−
	K6K-soil	+	−	+	+	+	−
	K6M-soil	+	−	+	+	+	−
Soil slurry[d]	M-Na					+	−
	K-Na					+	−
	K-soil					+	−
	K6K-soil					+	−
	K6M-soil					+	−

[a] K-Na, kaolinite homoionic to Na; K-Ca, kaolinite homoionic to Ca. See Table 1 for definitions of other abbreviations.
[b] +, utilization; −, no utilization (determined by measurement of O_2 uptake by the direct Warburg method).
[c] Mixed culture from a protein-enriched soil slurry incubated at 37°C in Davis citrate minimal medium for 50 h.
[d] Mixed culture from a garden soil (1:4 [wt/vol] soil-tap water) amended with free pepsin or with free or bound toxin from *B. thuringiensis* subsp. *kurstaki* and incubated at 25°C for 7 h.

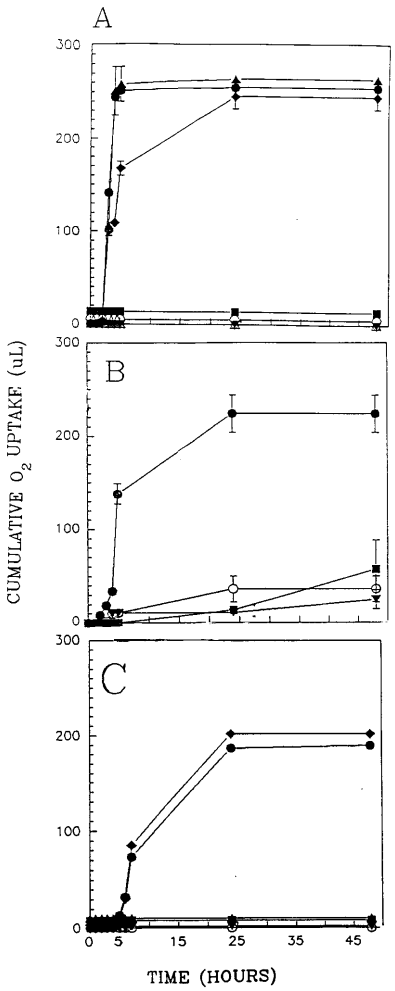

FIG. 4. Oxygen uptake by a mixed microbial culture from a protein-enriched soil slurry on pepsin (A and C; 200 μg/ml) or the toxin from *B. thuringiensis* subsp. *kurstaki* (A and C, 200 μg/ml; B, 100 μg/ml), free or bound on montmorillonite homoionic to calcium (0.25 mg/ml) or sodium (0.24 mg/ml) (A), kaolinite homoionic to calcium (0.43 mg/ml) or sodium (0.38 mg/ml) (B), or the clay-size fraction separated from K-soil, unamended (0.83 mg/ml) or amended to 6% (vol/vol) with kaolinite (K6K-soil [1.00 mg/ml]) or montmorillonite - (K6M-soil [0.38 mg/ml]). Data are expressed as means ± the standard error of the means, which is indicated when not within the dimensions of the symbols. ◆, pepsin; ●, toxin; ▼, toxin bound on montmorillonite (A) or kaolinite homoionic to calcium (B) or unamended K-soil (C); ■, toxin bound on montmorillonite (A) or kaolinite (B) homoionic to sodium or K6M-soil (C); ▲, pepsin plus 1.00-mg/ml montmorillonite homoionic to calcium (A) and toxin bound on K6K-soil (C); ○, control (no toxin, no clay, no microbes); ◇, control (no toxin, no clay, microbes); △, control (no toxin, no microbes, 1.00-mg/ml montmorillonite homoionic to calcium).

higher LC_{50} values than in soil continuously maintained at room temperature and the −33-kPa water tension), the toxin remained insecticidal after 40 days of incubation, regardless of how the soil was maintained.

DISCUSSION

The active toxins of *B. thuringiensis* expressed by transgenic plants and microbes may accumulate in soil if more toxins are produced than are consumed by insect larvae, degraded by microbes, or inactivated by sunlight. Excess toxins may accumulate after postharvest biomass is plowed into soil and the toxins bind on clays and other soil components, which renders them less available for biodegradation (13, 58–61, 71). Other proteins, peptides, amino acids, viruses, and DNA have also been shown to be protected against microbial degradation when bound on clays (23, 34, 53, 54). In this study, in which bound toxins were exposed to conditions that were more extreme than those that usually occur in soil in situ (e.g., microbes that utilize proteins readily and rapidly were enriched, whereas soil contains a diverse microbial community that is more oligotrophic and relies on numerous substrates for carbon and nitrogen), they were protected from microbial utilization and remained insecticidal, confirming that the bound toxins persist and retain insecticidal activity in soil (59, 60).

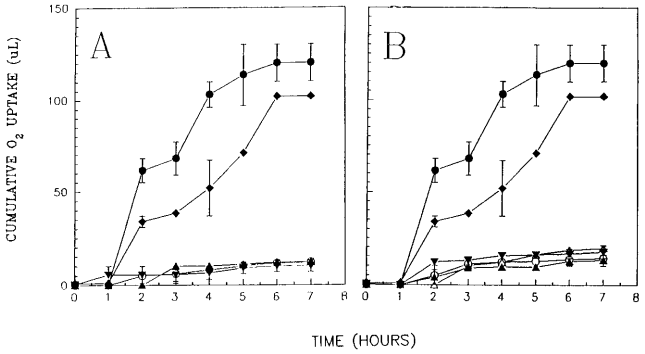

FIG. 5. Oxygen uptake by a mixed microbial culture from a soil slurry (1:4 fresh garden soil-tap water) on pepsin (100 μg/ml) or the toxin from *B. thuringiensis* subsp. *kurstaki* (100 μg/ml), free or bound on montmorillonite or kaolinite homoionic to sodium (A) or the clay-size fraction separated from K-soil unamended or amended to 6% (vol/vol) with kaolinite (K6K-soil) or montmorillonite (K6M-soil) (B). Data are expressed as means ± the standard error of the means, which is indicated when not within the dimensions of the symbols. ◆, pepsin; ●, toxin; ▼, toxin bound on montmorillonite (A [0.28 mg/ml]) or K-soil (B [0.41 mg/ml]); ▲, toxin bound on kaolinite (A [0.70 mg/ml]) or K6K-soil (B [0.50 mg/ml]); △, toxin bound on K6M-soil (0.19 mg/ml); ○, control (no toxin, no clay, no microbes).

Some of the mechanisms by which proteins bound on clays resist utilization or inactivation by microbes include the following. (i) There may be intercalation of swelling 2:1-layer clays, such as montmorillonite, which renders proteins inacces-

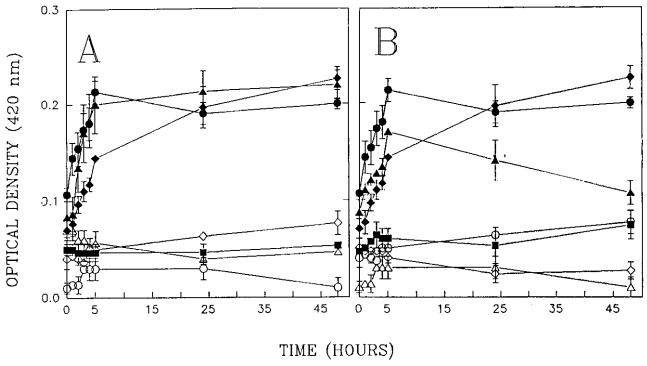

FIG. 6. Growth of a mixed microbial culture from a protein-enriched soil slurry on pepsin (200 μg/ml) or the toxin from *B. thuringiensis* subsp. *tenebrionis* (200 μg/ml), free or bound on montmorillonite homoionic to sodium (A [3.55 mg/ml]) or the clay-size fraction separated from K-soil (B [3.81 mg/ml]). Data are expressed as means ± the standard error of the means, which is indicated when not within the dimensions of the symbols. ◆, pepsin; ▲, pepsin plus montmorillonite (A [1.00 mg/ml]) or clay-size fraction (B [1.00 mg/ml]); ●, toxin; ■, toxin bound on montmorillonite (A) or clay-size fraction (B); ○, control (no toxin, no clay, and either no microbes [A] or microbes [B]); ◇, control (no toxin, no clay, microbes); △, control (no toxin, 1.00-mg/ml montmorillonite, microbes [A], or 1.00-mg/ml clay-size fraction, no microbes [B]).

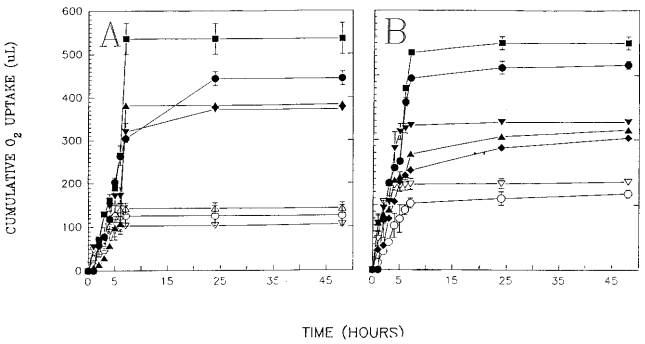

FIG. 7. Oxygen uptake by a mixed microbial culture from a protein-enriched soil slurry on the toxin from *B. thuringiensis* subsp. *kurstaki* (200 μg/ml), free or bound on montmorillonite (0.40 mg/ml) or kaolinite (0.40 mg/ml) homoionic to sodium (A) or the clay-size fraction separated from K-soil (B), unamended (0.66 mg/ml) or amended to 6% (vol/vol) with kaolinite (K6K-soil [0.48 mg/ml]) or montmorillonite (K6M-soil [0.30 mg/ml]), as a sole source of carbon, nitrogen, or carbon plus nitrogen in the presence or absence of glucose (255 μg/ml). Data are expressed as means ± the standard error of the means, which is indicated when not within the dimensions of the symbols. ●, toxin; ■, toxin plus glucose; ▼, toxin bound on kaolinite plus glucose (A) or K-soil plus glucose (B); ▲, toxin bound on montmorillonite plus glucose (A) or K6K-soil plus glucose (B); ◆, toxin bound on K6M-soil plus glucose; ▽, toxin bound on kaolinite (A) or K-soil (B); △, toxin bound on montmorillonite; ○, control (no toxin, no glucose, no clay, microbes).

sible to proteolytic enzymes. (ii) Terminal amino acid residues necessary for initiation of cleavage by peptidases may be involved in binding and, therefore, inaccessible. (iii) Binding may so alter the conformation of proteins that the peptide bond may be nonsusceptible to peptidases. (iv) Substances in soil (e.g., ions, humic substances, and other organic molecules) may bind on or become associated with clay-protein complexes and render the protein inaccessible (53). The toxins only partially intercalated montmorillonite (as indicated by X-ray diffraction analysis), and their conformation (as indicated by infrared and immunological analyses and electrophoretic mobility) was not changed by binding (61). Therefore, bound toxins may have been protected from microbial utilization by the inaccessibility of amino acid residues and, in the soil studies, by the binding of substances in soil on the clay-toxin complexes.

The mechanisms by which clay-bound toxins kill susceptible larvae, even though the toxins were resistant to biodegradation, are not known. The binding of proteins on clay is primarily by H bonds, and segments of the proteins are constantly detaching and reattaching (53). Consequently, toxins could move from the clay to receptors on the epithelium of the larval gut when an end of the toxins detaches from clay, especially if the receptors have a higher affinity (specific binding) for the toxins than does the clay (nonspecific binding) (53). In addition, pH-dependent changes in the conformation of the toxins, which may affect the relative susceptibility of their C and N termini to cleavage by proteases, may be involved (11).

Regardless of the mechanism by which clay-bound toxins become resistant to biodegradation and retain insecticidal activity, the toxins from transgenic plants and microbes may accumulate in soil and, thereby, may enhance the development of insect resistance and pose a potential hazard to nontarget organisms. Although numerous strategies have been developed to manage or prevent the development of insect resistance resulting from the planting of transgenic *B. thuringiensis* crops (e.g., crop rotation; maintaining a supply of susceptible insects by refuges or immigration; estimation and prediction of resistance through the use of diagnostic tools, monitoring, and models; reduction in the selection for each mortality mechanism by decreasing the intensity of selective pressures; and diversification of mortality sources, such as the use of different or multiple Cry proteins) (4, 29, 40, 55), none has apparently considered and integrated the potential persistence of the toxins as a result of binding on clay and other soil particles, which could circumvent all the strategies. For example, rotation, in which a plant containing the first toxin (e.g., CryI) would be replaced by another plant containing a different toxin (e.g., CryII), may be ineffective if the first toxin bound on soil particles persists and the larvae, which may be sensitive to both toxins, are simultaneously exposed to both, thereby accelerating the development of resistance to both.

The results of the current studies indicate that the toxins

TABLE 3. LC_{50} of the toxin from *B. thuringiensis* subsp. *kurstaki*, free or bound, for *M. sexta* larvae[a]

Culture addition and LC_{50} (μg/100 μl) of free toxin	Clay or soil fraction bound[b]	LC_{50} (μg/100 μl) of bound toxin	RP[c]
None (control)			
1.3 ± 0.05[d]	M-Na	1.6 ± 0.45	0.8
	K-Na	1.2 ± 0.35	1.1
0.5 ± 0.27[e]	M-Na	0.4 ± 0.11	1.3
	K-Na	0.4 ± 0.01	1.3
0.1 ± 0.10[f]	K-soil	0.2 ± 0.09	0.5
	K6K-soil	1.4 ± 0.12	0.1
	K6M-soil	1.9 ± 0.13	0.1
20.0 ± 7.90[g]	M-Na	40.0 ± 6.00	0.5
	K-Na	23.0 ± 11.50	0.9
Microbe or enzyme			
Mixed			
4.6 ± 0.19[d]	M-Na	1.0 ± 0.35	4.6
	K-Na	0.6 ± 0.01	7.6
1.0 ± 0.15[e]	M-Na	0.5 ± 0.26	2.0
	K-Na	0.7 ± 0.19	1.4
6.5 ± 2.50[f]	K-soil	1.0 ± 0.01	6.5
	K6K-soil	4.0 ± 0.26	1.6
	K6M-soil	5.1 ± 2.10	1.2
S. hygroscopicus			
263.0 ± 87.00	M-Na	23.0 ± 0.50	11.4
	K-Na	47.0 ± 15.50	5.6
S. griseus			
117.0 ± 31.00	M-Na	105.0 ± 18.51	1.1
	K-Na	48.0 ± 12.50	2.4
Pronase E (1 mg/ml)			
91.0 ± 10.50	M-Na	43.0 ± 6.50	2.1
	K-Na	18.0 ± 2.00	5.1

[a] The LC_{50} of toxin, free or bound on homoionic clays or the clay-size fraction from soil, after exposure for 3 days at 37°C to a mixed culture of microorganisms from a protein-enriched soil slurry or for 7 days at 25°C to pure cultures of *Streptomyces* spp. or pronase E. LC_{50} values are means ± standard error of the means.

[b] See Table 1 for definitions of abbreviations.

[c] RP is measured as the LC_{50} of free toxin/LC_{50} of bound toxin. An RP of 1.0 indicates no difference in potency between the free and bound toxin, an RP of >1.0 indicates a greater potency of the bound toxin, and an RP of <1.0 indicates a lower potency of the bound toxin.

[d,e,f] Control for the corresponding mixed cultures.

[g] Control for the *Streptomyces* spp. and pronase.

TABLE 4. LC_{50} of the toxin from *B. thuringiensis* subsp. *kurstaki*, free or bound on homoionic clays, for the larvae of *M. sexta*[a]

Treatment	LC_{50} (μg/100 μl) ± SE[b]			RP of toxin bound on[c]:	
	Free toxin	Toxin bound on			
		M-Na	K-Na	M-Na	K-Na
Stock[d]	0.7 ± 0.01	0.7 ± 0.01	0.5 ± 0.0		
Room temp[e]	5.5 ± 1.55	3.0 ± 0.90	3.9 ± 1.00	1.8	1.4
Air dried and rewetted[f]	11.2 ± 3.30	17.2 ± 5.36	17.0 ± 1.54	0.7	0.7
Frozen and thawed[g]	24.0 ± 2.25	13.4 ± 0.34	7.6 ± 0.70	1.8	3.2

[a] LC_{50} was measured after incubation of the toxin for 40 days in K-soil (100 μg of toxin/g of soil) continuously at the −33-kPa water tension and room temperature, which was then alternately air dried and rewetted or alternately frozen and thawed.

[b] M-Na, montmorillonite homoionic to sodium; K-Na, kaolinite homoionic to sodium.

[c] See Table 3 for details.

[d] Free toxin was stored as aliquots at −20°C, and bound toxin was stored as a pellet at 4°C; both were assayed for their LC_{50} immediately after purification or binding on clay minerals.

[e] Maintained at room temperature at the −33-kPa water tension.

[f] Maintained at room temperature at the −33-kPa water tension for 7 days, air dried for 7 days, and rewetted and maintained at the −33-kPa water tension for 7 days. This cycle was repeated twice.

[g] Maintained at room temperature at the −33-kPa water tension for 7 days and then at −20°C for 7 days. This cycle was repeated three times.

from *B. thuringiensis* subspp. *kurstaki* and *tenebrionis* may accumulate in soil and remain insecticidal as the result of their resistance to microbial utilization when bound on clay. This may be a mechanism by which other products of novel genes (e.g., enzymes, antibodies, vaccine epitopes, and other insecticides [7, 9, 12, 26, 30, 38]) could persist and retain activity in the environment into which they are introduced. The potential of bioactive proteins, viruses, and DNA persisting in the environment as a result of binding on clay and other soil particles supports a growing body of evidence that indicates there may be risks in releasing genetically modified organisms to the environment (54, 77). Not only must this mechanism of persistence be incorporated in future management plans to reduce the development of insect resistance to the toxins from *B. thuringiensis*, but studies are needed to evaluate the impacts of persistence by this mechanism in the field.

ACKNOWLEDGMENT

These studies were supported, in part, by grant R816638 from the U.S. Environmental Protection Agency.

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